UNIVERSITAT DE BARCELONA

Final Degree Project

Biomedical Engineering Degree

“ Optimization of an obtaining nanotubes process ”

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ABSTRACT

Because of the aging of the population and the increase medical interventions with prothesis, the use of metallic implants is increasing exponentially rising the need to improve their lifespan and biocompatibility. So that, it is important to improve how the material interact with the tissues of the body. That is the reason why, this project has aimed at making surface modifications of titanium implants to increase their bioactivity.

Specifically, this study has been focused on making surface modifications using an electrochemical anodization process of a particular titanium alloy, Ti-6Al-7Nb, to generate nanotubes. Through the electrochemical anodization process, a titanium dioxide (TiO_2) layer is generated. This oxide layer provides the surface with a high corrosion resistance that protects and prevents the release of ions from the implant to the body. Also, this TiO_2 layer is a bioactive ceramic material which will favor cell adhesion and proliferation. Therefore, this project has consisted of three steps: optimize the conditions to create nanotubes on an industrial level, characterize the coatings made through imaging with different microscopic techniques and finally validate the sample bioactivity by cellular viability tests.

Last but not least, it is important to note the methodology behind this degree thesis. First the background of titanium and its alloys as a biomaterial is explained, as well as the evolution of implants and current techniques available to improve the bioactivity of surfaces. In addition, a market study has been carried out and both the technical and economic feasibility of the project have been studied. Finally, the current regulations on medical devices have been considered. To sum up, it has been possible to establish an overview of the aspects necessary to carry out modifications of surfaces of medical implants.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

1. INTRODUCTION

1.1. Objective

Nowadays, the use of metallic implants is increasing exponentially rising the need to improve their lifespan and biocompatibility. Related to this fact, the project is based on making surface modifications of titanium implants to improve their bioactivity. Specifically, the study will focus on surface modifications using an electrochemical anodization process of a particular titanium alloy, Ti-6Al-7Nb, to generate nanotubes on the surface.

In addition, cell toxicity and viability studies will be done to determine the cytotoxic effects of the samples in the body. The main requirement is that the procedure employed must be industrially reproducible. Therefore, the objectives of this project could be defined as three different:

- Optimizing the conditions to create nanotubes on an industrial level.
- Characterization of the coatings made.
- Validation of the bioactivity by cellular viability tests.

1.2. Methods and memory structure

During this study, the first step was to carry out an extensive bibliographical research to know the properties that characterize titanium implants, and to determine which modifications will improve their bioactivity and biocompatibility. Detailed information was obtained through the NCBI website, which allows to limit and customize the search according to the interests of the study. Once the project was studied and approved, the practical and experimental took place in Materials Science and Chemistry Physics department at the Faculty of Chemistry of the University of Barcelona.

On the other hand, the project memory follows the structure shown in the index. The first part of the memory is the definition of the objectives of the work, followed by the historical evolution of the implants, the current situation in the market and possible modifications that can be made to improve bioactivity and biocompatibility.

After the description of the state of the art the memory continues with the practical part, where is located the experimental methodology, the characterization methods used, the results obtained and the conclusions of the project. Last but not least, the feasibility of the project was analyzed, as well as the time it took to carry it out.

1.3. Process description

Prostheses need to have specific mechanical properties, because depending on the function it may have to withstand high stresses. In addition, prosthetics were formerly sought to be bioinert, but over time, advances in biomaterials engineering have led to the search for solutions to improve biocompatibility, and as a consequence, bioactivity. These properties can be achieved with the correct election of the material used and different surface modifications. The selected material for this project is Ti-6Al-7Nb due to the wide use of titanium alloys in implantology. Then, a modification to the surface was made with the aim of improving bioactivity. By modifying roughness and surface area, it can be provided a more suitable substrate for protein adsorption and cell growth, accelerating the bone regeneration process and improving osseointegration of implants. In this case, the surface modification was carried out by an electrochemical anodization of titanium substrates, to create a nanometric titanium dioxide (TiO_2) coating with a nanotube shape. This process will improve adhesions because morphology of these nanometric structures are similar to those of the bone [1].

1.4. Scope and reach

This project is carried out to make a comprehensive study on how implanted prostheses can be improved by modifying their surface by a specific treatment such as the anodizing. It is located in the field of biomedical engineering, specifically in materials and biomaterials engineering. In addition, within the research line of the department, it pursues its subsequent implementation in the industrial sector.

2. BACKGROUND

2.1. Biomaterial background

The RAE define biomaterial as "Material tolerated by the organism, used for prosthetics and other purposes", but their first uses date back to the ancient Egyptian. Remains of an Egyptian prosthesis were found, it was made of wood and leather fixed by a gold thread. So, it is a fact that with the advancement in medicine, technology and engineering, biomaterials used in prosthetics implants have evolved a lot since then.

Titanium and its alloys are a very interesting biomaterial for its mechanical properties, corrosion resistance, processability and availability [2]. Although, it was not until at the end of the 50s that its biocompatibility and osseointegration properties were observed and consequently, it began to have a medical use [3]. The great biocompatibility of titanium alloys arrays in the creation of a coherent nanometric titanium oxide coating on their surface that protects the implant from corrosion processes. Titanium was discovered by William Gregor in 1791 and in 1795 named because of the titans of Greek mythology by Martin Heinrich Klaproth.

2.1.1. History of titanium as a biomaterial - medical implants

Three types of generations can be distinguished in the evolution of implants. The first generation was characterized by searching for implants with physical properties similar to the tissue in which they were implanted, having low toxicity and being bioinert. The second generation of implants was based on implants with a bioactive character by surface modifications. The third generation of implants includes improvements focused on a long-term improvement of implants by promoting a specific cellular response at the molecular level.

- <u>First generation of medical implants</u>

The first generation of medical implants is defined as inert prostheses which have physical properties similar to the tissue in which they were implanted. Inert means lifeless. The first prostheses made with titanium alloys, were produced in the 80s and the alloy used was Ti-6Al-4V. These alloys are considered of double phase because they combine crystalline phases such as α-phase and β-phase at room temperature. The mechanical properties of the bulk material are determined by the proportion of these phases, for that reason, it is possible to custom its properties by modifying the proportion of the phases or the alloying elements.

Stabilization of the beta phase using alloying elements such as Vanadium (V) is one of the most important features of these alloys. But a high number of scientific studies founded a segregation of high concentrations of vanadium oxide (V_2O_5) around the implant. The problem with this is that high concentrations are toxic to the body, being able to produce carcinogenic effects or interference with systematic reactions of the body causing implant failure [2].

Because of this problem, different elements began to be investigated to develop titanium alloys with beneficial properties. These elements include Niobium (Nb), Tantalum (Ta), Zirconium (Zr) and Molybdenum (Mo). All of them, are beta-phase stabilizing elements that can replace vanadium in the alloy.

For that reason, Ti-6Al-7Nb alloys were developed, in which vanadium (V) was replaced by Niobium (Nb), the microstructure and mechanical properties of the alloy are similar, however corrosion resistance and biocompatibility were improved [2]. Ti-6Al-7Nb have a low Young module, which reduce mechanical tensions and prevents osteolysis (bone loss).

Table 1, shows a comparison of mechanical properties between titanium alloys and bone.

	Young Modulus (GPa)	Compressive Strength (MPa)	Shear Modulus (GPa)
Ti-6Al-4V	111-119	$(1{,}10 - 1{,}15) \cdot 10^3$	40-45
Ti-6Al-7Nb	100-110	$(1{,}07 - 1{,}09) \cdot 10^3$	36-41
Bone	17-22	114-167	3.3-6

Table 1. Mechanical properties of titanium alloys and bone.

** All properties were taken from CES Edupack

- Second generation of medical implants

To improve the survival of implants, coatings with bioactive materials can be carried out or a nano-roughness surface can be carried out to improve material-host interaction. The second generation of implants is based on this, implants with a bioactive character because of surface modifications. Bioactivity is the ability of a material to interact with the tissues of the body [4] and osseointegration is defined as a direct, structural, and functional connection between the bone and the surface of an implant subjected to functional load [5].

For many years, prostheses have failed because the interface between the implant and the bone was lost, so a deficiency in osseointegration occurred. Then, they began to take an interest in treatments that would cause modifications in the surface of these implants. Modifying roughness and surface area improves the biocompatibility of the metallic implants. The biocompatibility is referred to the quality that has a biomaterial to generate an acceptable biological response during the time of contact with the organism [6].

The coating of a bioinert material, such as titanium, with a bioactive ceramic layer, aims to modify the morphology and surface composition to increase the survival rate of the implant. The oxide layer formed have properties such as a high corrosion resistance that protects and prevents a further oxidation. The transfer of ions from inside the implant to the body, may favor cell growth depending by the ions and materials used in the implantable [7]. But the releasing of ions from the implants can also being toxic for the surrounding tissues, depending of the type of ion or if they are in a high concentration.

The most commonly bioactive ceramics used as a coating are titanium dioxide and hydroxyapatite because of their high bioactivity, that increases osseointegration and reduces the risk of failure and loss of the implant. Specifically, titanium alloys can form a protective layer of titanium dioxide that protects the implant from the physiological medium in which it is introduced; this is why has low corrosion. Due to the protection generated by this ceramic layer of TiO_2, and depending on the ion release the implant can be considered bioactive.

Among the common surface treatments used to obtain coatings are plasma spray, sandblasting, acid treatment or sintering with spherical powders. All of them were done on a micrometric scale [2]. As regards to coatings on the nanometric scale, there are alkaline oxidation where a subsequent heat treatment can be performed and an electrochemical anodization with which they obtain a nanometric structure of nanotubes.

2.1.2. Titanium alloys

Titanium has allotropic capacity; meaning that its crystalline structure is transformed depending on whether it is above or below 882.5 ºC which is the transformation temperature. Below this temperature is the α-phase at which a compact hexagonal structure (HCP) follows. Above the processing temperature is β-phase which has a body-centered cubic crystalline structure (BCC) [8]. Figure 1 shows the difference between the crystal phases.

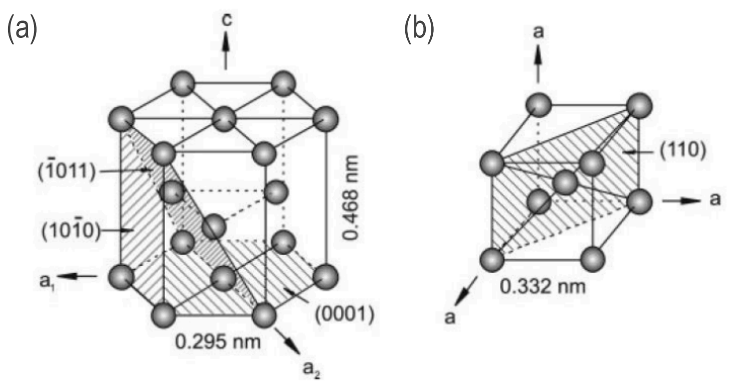


Figure 1. (a) Titanium α-phase structure - HCP. (b) Titanium β-phase structure - BCC [8].

The allotropic capacity and the presence of two different crystalline structures determines the mechanical properties of titanium. It can be differentiated between three types of alloys: type α, type α+β, and type β. In addition, the transformation temperature can be modified by the presence of alloying elements that will stabilize α-phase or β-phase depending on whether they raise or decrease the transition temperature [8].

- Type α titanium alloys generally contain Aluminum (Al) and Tin (Sn) as they are α-phase stabilizing elements. They are also characterized by providing greater hardening and fatigue resistance [9].
- Type β titanium alloys are usually made up of Vanadium (V) and Molybdenum (Mb) [9]. They have a high resistance to fatigue corrosion and a low elasticity module [2].
- Type α+β titanium alloys are characterized by an α-phase stabilizing element such as Aluminum (Al) and a β-phase stabilizing element such as Vanadium (V). They are ductile and fatigue-resistant alloys. Moreover, they have good fracture toughness [9].

2.2. Current situation

Natural tissues are so complex. Although Young's modulus (E) of metals used in orthopedics implants are about 10 times higher than the Young's modulus of natural bone, metals are still being used, because there is no synthetic tissue that reproduces the mechanical characteristics of natural bone tissues.

To improve long-term survival and promote specific cellular response at a molecular level, it is essential to ensure optimal conditions to achieve osseointegration in the shortest period of time. Osseointegration is determined by the degree of bioactivity and biocompatibility of the implant. By increasing the bioactivity of the implant, the interaction of implant-cells osteoprogenitors can be improved and the recruitment of bone-promoting cells is increased [2].

In implants of third generation both bioactivity and biodegradability are combined. The bioactivity of surfaces is carried out by specific biomolecules that act as a guide and cellular stimulation to achieve a specific response. The behavior of the cells will depend on the biomolecules attached to the surface of the implant. On the other hand, if an inorganic (or biodegradable) phase is added, the mechanical behavior of the structure is also modified [10].

Nowadays, research is focused on HA, microspheres, nanocrystalline structures, organic-inorganic composites, fibers, three-dimensional ACP scaffolds, tuned porosity microstructures and hierarchically organized structures [10].

2.3. State of technology

Currently, the most used technology belongs to the second generation of medical implants. Performing surface treatments to increase the roughness of the sample or create an oxide layer on the implant.

Among the different methods or treatments to increase the roughness of the surfaces of implant surface, it is found the plasma spray, sandblasting, acid treatments and so on [2]. These methods are used in the second generation of medical implants to increase the bioactivity of the surface. These types of modifications improve bone growth and implant viability.

The plasma spray technique can produce a layer of between 30 and 50 µm with thickness increasing the roughness and the surface of the implant. It is based on making projections of materials in the form of finely divided molten particles, on a properly prepared substrate [11].

Sandblasting is based on projecting abrasive particles of alumina (Al_2O_3), titanium dioxide (TiO_2) or hydroxyapatite, at high pressure on the surface to achieve the roughness of about 10 µm [11].

Acid treatments are used to obtain surfaces with roughness between 0.5 and 4 µm and help to improve osseointegration. The surface is attacked with strong acids such as hydrochloric, sulfuric, nitric or hydrofluoric for short periods of time [11].

As regards the surface treatments to achieve an oxide layer and improve bioactivity, chemical vapor deposition (CVD) and electrochemical anodization are differentiated [2].

CVD consists of the reaction of a mixture of gases inside a reactor to form a thin layer of a material, in this case an oxide layer [12].

Electrochemical anodization treatments based on an oxidation-reduction reaction. The process consists of using a potential difference between the cathode and the anode (titanium sample), generate a titanium dioxide layer on the surface of the implant. This coating acts increasing adhesion and cell growth on surfaces. Finally, heat treatments can be done to modify the crystalline structure of oxide surfaces obtained by electrochemical oxidation. A crystalline structure of titanium nanotubes is obtained and it help cell growth: improving the adhesion, proliferation and differentiation of osteoblasts [2].

3. MARKET ANALYSIS

3.1. Sectors targeted

Last years there has been an aging of the population. In fact, Spanish data published in 2019 reflects an increasing aging population. Currently almost 20% of the population are over 65 years old, while if we go 30 years back, in 1990, the percentage of the population over 65 years was about 13% [13]. The aging of the population has social and economic consequences. Medical treatments are concentrated in the higher age groups due to the increase in the life expectancy of the population.

Figure 2 shows the number of medical interventions performed to treat fractures, per 10,000 inhabitants, depending on the age of the patients [2].

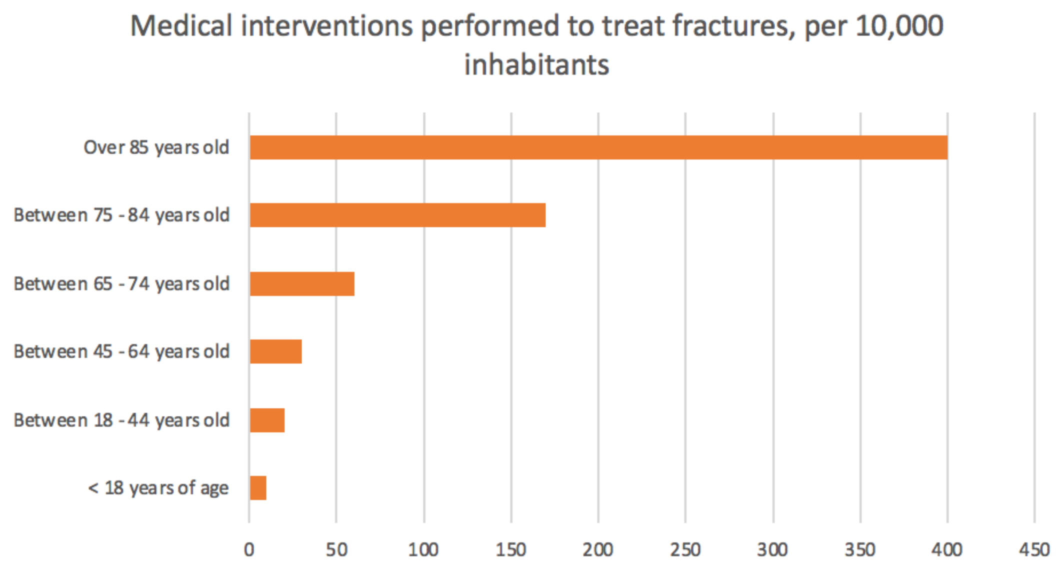


Figure 2. Medical interventions performed to treat fractures, per 10,000 inhabitants, depending on the age of patients. Adapted from: National Center for Health Statistics (2015)

This project is aimed at research to improve orthopedical implants and consequently, for everyone who need an implant either spinal or dental. Therefore, it is necessary to innovate in this field because in the future, the population will continue to aging and improvements will be needed.

The project seeks to improve the biocompatibility and osseointegration of metallic implants. In addition, research is based on methods that can be reproduced industrially, so it is also focused on the massive production of these implants.

3.2. Historical market evolution of titanium

Since titanium was discovered in 1971, it took a century isolated it because in nature it is found in a compound with oxygen or iron. During the 1950s, titanium was started to be used for military aviation, so the Soviet Union used this material heavily for military applications and submarine development. In the decade of the 50s United States also began to support its commercialization. Later during the Cold War, the United States generated a large metallic titanium reserve.

In 2006, almost 30% of titanium production was carried out by the Russian company. The United States began investing in a process of producing the powdered material for use in different fields. In 2015 production had already spread throughout China, Japan, Russia, Kazakhstan, the United States, Ukraine and India.

Nowadays, titanium is used in steel alloys to reduce grain size and as a deoxidizer, and in stainless steel alloys to reduce its carbon content. Alloys with other elements are also common to obtain different properties. Knowing this, between 90% and 95% of the consumption of this material is concentrated in the paint industry (pigments), in polymers, in the paper industry or in the ceramic industry. And only 5% to 10% is concentrated in the metallurgical industry or for the production of metal titanium and titanium alloys.

3.3. Historical market evolution of implants

As has been developed previously, the population in recent years has aged. Moreover, the development of new products and the increase in healthcare spending in developed countries has led to a significant expanded global market for medical implants. Global health spending in a decade is estimated at the order of trillions. These is why the implant market has also had a significant highlight in recent years.

Nowadays, the medical implants market is divided by company, type, application and geographical location. Depending on the types of implants, the market is divided by orthopedic, cardiac, spinal, dental, ophthalmic and cosmetic medical implants. Specifically, knee, hip, dental and spine implants represent more than half of the world's production of implants [14].

Implant surface coatings have also segmented the market. Depending on the type of coating or modification, the implant market can be differentiated into passive surface finishes (PSM), active surface finishes (ASM) and antibacterial coatings (LCC) [15].

3.4. Future market outlook

Analyzing the current situation, the future market is focus on improving existing prostheses, making it more durable over time and with better mechanical properties. In addition, the research and implementation of surface modifications and implant coatings will be the key point to achieve bioactive orthopedical implants, improving osseointegration and adding antibacterial properties to avoid infections.

On the other hand, the development of 3D printing of medical implantable has great prospects in the future. The success of this technique lies in the fact that personalized implants can be made. So on, it improves recovery and makes easier complex surgical cases. Nowadays, 3D printing of orthopedic implants is possible thanks to additive manufacturing (AM) that is another way to fabricate metallic products, in fact it allows to make net-shape geometry high fatigue strength and corrosion resistant parts [16].

4. CONCEPTION ENGINEERING

4.1. Study of solutions

There are different ways to improve the bioactivity of implants. One of the options is to focus on achieving roughness on the surface to increase cellular adhesion and future proliferation. The second option consists of oxidation on the implant surface in which a titanium dioxide layer is created on the surface.

First, techniques such as plasma spray, sandblasting, acid treatments and so on, are methods to improve osseointegration because cause a rougher surface in the microscale. However, to obtain better biocompatibility and osseointegration it is necessary to create a bioceramic coating.

To improve implants methods as alkaline-thermal oxidation, electrochemical anodization or coatings with hydroxyapatite can be used.

The first one consists of carry out an alkaline oxidation to increase the thickness of the oxide layer performing an immersion in alkaline solutions such as NaOH or KOH. Then, the process is continued thermally heating the titanium implant at about 600-750 ºC in an oxidizing atmosphere. This causes the formation of a titanium dioxide layer on the surface.

On the other hand, the electrochemical anodization consists of using a potential difference, generate a titanium dioxide layer with a crystalline structure of titanium nanotubes on the surface of the implant.

Another strategy is to coat titanium with polymeric substances that resemble the extracellular matrix or may even help to bind proteins that can improve interaction with osteoblasts.

Also, coatings of hydroxyapatite can be used. It consists of using a hydroxyapatite (HAp) layer to improve adhesion. This bioceramic material can be applied from a sol-gel source by spin-coating at 1500 rpm for 20s followed by heat treatments at different temperatures between 450 and 1200 ºC [1].

4.2. Proposed solution

Of all the options seen before, the project is focus on the electrochemical anodization of Ti-6Al-7Nb. The anodizing process is an electrochemical oxidation process where a nanostructure morphology as nanotubes can be achieved. The implant acts as an anode and it is immersed in an electrolyte, then an electric current is applied, so that the oxidation of the implant surface is performed.

Nanotubes are mainly formed by two processes: chemical attack and chemical dissolution. First, an initial oxide layer is formed on the sample surface by the interaction of the alloy components with the O^{2-} ions. Then, due to the F^- ions located in the electrolyte used for anodizing, a localized dissolution of the F^- ions occurs, producing pores on the surface. These pores increase as the oxide layer moves into the sample. In this way, nanotubes are differentiated from others and the nanotubular structure is produced on the surface [7].

Previously, studies had been conducted at very high potentials (~200 V) for short periods of time. In this way nanotubes could be created but it was not possible to control their morphology. For that reason, the proposed solution is based on working at low potential and for longer times controlling the creation of these nanotubes [17].

In addition, it is possible to realize a subsequent heat treatment to stabilize a specific crystal phase of titanium oxide such as anatase or rutile. In this project was not possible to optimize this step but a future work is necessary to continue studying the effect of a thermal treatment on the nanotube's morphology and crystal structure and to understand the effect of a thermal treatment in the bioactivity of the samples.

5. DETAILED ENGINEERING

5.1. Materials and Methods

5.1.1. Reagents

For cleaning protocol and anodizing has been used surfactant sodium dodecilbenzenesulfonate (Sigma-Aldrich with number CAS:25155-30-0), NaOH (Sigma-Aldrich CAS: 1310-73-2), Na_2CO_3 (Sigma-Aldrich CAS: 497-19-8), HNO3 69 wt% (Sigma-Aldrich CAS: 7697-37-2), HF 40 wt% (Carlo Erba CAS: 7664-39-3), ethylene glycol (Sigma-Aldrich with CAS number: 107-21-1) and NH_4F 40 wt%(Carlo Erba CAS: 12125-01-8).

5.1.2.Sample preparation

The samples that have been worked with are 2 mm thick Ti-6Al-7Nb alloy plates. The titanium plate has been cut into 0.5 x 1 cm2 pieces. A careful metallographic preparation was done to guarantee the roughness of the sample will not affect to the anodization process. The sample was grinded with SiC paper (P1200, P2500 and P4000). Then, a mechanical polishing has been done with colloidal diamond paste of 6 mm and 1 mm, respectively. An example of a sample before anodizing can be observed in Figure 3.

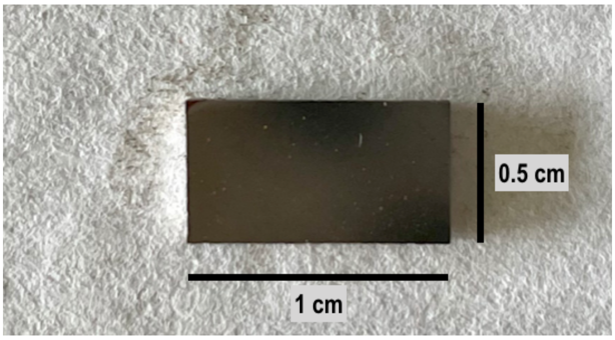


Figure 3. Ti-6Al-7Nb alloy plate.

5.1.3. Cleaning protocol

To begin with the characterization of the samples, the first step has been to prepare the samples for the anodizing. Preparation consists of cleaning up Ti-6Al-7Nb samples using a cleanup protocol consisting of three steps.

1. First, the protocol is started by inserting the sample into a beaker with Mili-Q water and sodium dodecilbencesulfonate. It is left shaking for five minutes (Figure 4) and then three washing cycles by support are performed to rinse the sample and remove all

traces of soap. This first step is very important to remove all the grease that may be on the homogeneous surface.

2. Electrolyte cleaning is then performed at 5 V for 2 minutes and 30 seconds. The degreaser used consists of 35 wt% of NaOH and 65 wt% of Na_2CO_3 (Figure 5).
3. Finally, a chemical pickling is performed for 10 minutes in which the sample is inserted and is completely submerged. The compound consists of 30 v/v% HNO_3, 3 v/v% HF and 67 v/v% H_2O.

Figure 4. First step of the cleaning protocol. Mili-Q water with sodium dodecilbenzenesulfonate.

Figure 5. Electrolyte cleaning. The voltameter can be observed together with the degreasing solution.

5.1.4. Anodizing

For this study, anodizing has been performed using a device that allows the sample to be rotated in circles around a circular cathode submerged in an electrolyte (Figure 6). The objective of the assembly is to have agitation to minimize the concentration of substances on the surface that prevent homogeneous anodizing.

Figure 6. Rotating equipment with magnetic stirrer as a stand for anodizing.

Anodizing has been carried out on an electrolyte whose solution consisted of 0.1 v/v% NH_4F, 2 v/v% H_2O and 97.9 v/v% ethylene glycol.

First, samples were anodized at constant voltages of 15 V, 30 V, 45 V and 60 V for 30 minutes, 60 minutes and 120 minutes. And once one of the conditions had been chosen, samples were anodized at constant voltage of 60 V for an hour.

15 V	30 min	30 V	30 min	45 V	30 min	60 V	30 min
	60 min		60 min		60 min		60 min
	120 min		120 min		120 min		120 min

Figure 7. Working conditions at ethylene glycol.

5.1.5. Sample code

The code used for the nomenclature of each sample has been the following:

TiOx_AN_E2_POTENTIAL_MINUTES

- 'TiOx' due to titanium oxidation.
- 'AN' because it is done by anodizing.
- 'E2' due to the electrolyte used, in this case ethylene glycol.
- 'POTENTIAL': 15 V, 30 V, 45 V or 60 V.
- 'MINUTES': 30', 60' or 120'.

5.2.Characterization

5.2.1. Confocal microscope

The topography of the sample surface as well as roughness measurements were determined with Confocal Microscopy. A confocal microscope of the Sensofar trademark was used (Figure 8). Measurements were made with the objective lens of x150. The samples were measured by scanning from top to bottom in an area of 768 x 576 pixels, equivalent to 84.83 x 63.60 m ^{2}.

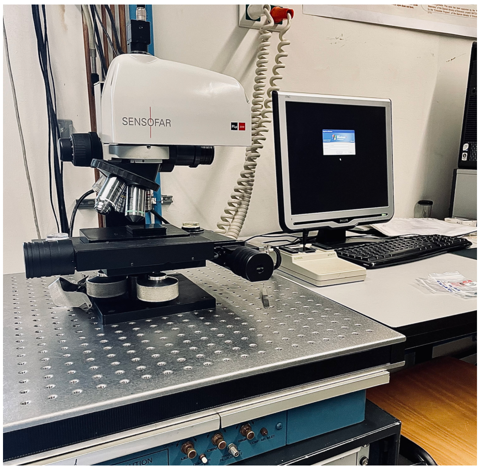


Figure 8. Confocal microscope.

5.2.2. Field Emission Scanning Electron Microscope (FESEM)

To determine the morphology and surface composition of anodized samples, FESEM JEOL J-7100 equipment (Figure 9) with a potential of 30 KV was used, working with secondary electron images and an X-ray detector (EDS) for composition determination. In addition, we will also observe the samples crosswise with this equipment. FESEM has been used for the high resolution it offers.

Figure 9. Scanning Electron Microscope.

5.2.3. Image processing and Surface Roughness

The processing of images has been carried out using the Gwyddion software. This program is a multimodular software used for data visualization and analysis [18]. In this project specifically, it has been used for the processing of images extracted from the confocal microscope. Important data have been obtained for morphological characterization, such as the roughness of the samples or the diameter of the nanotubes created.

The software contains multiple functions for the post-processing of the images. In order to analyze the images made with the confocal microscope, a protocol was followed. First, the measurements were scaled to the image. The image was scale before to proceed to extract the necessary data for further analysis. The protocol used was the same for all images, as is shown in Figure 10, the roughness data was extracted from a diagonal line obtaining the roughness parameters together with their corresponding profile graph.

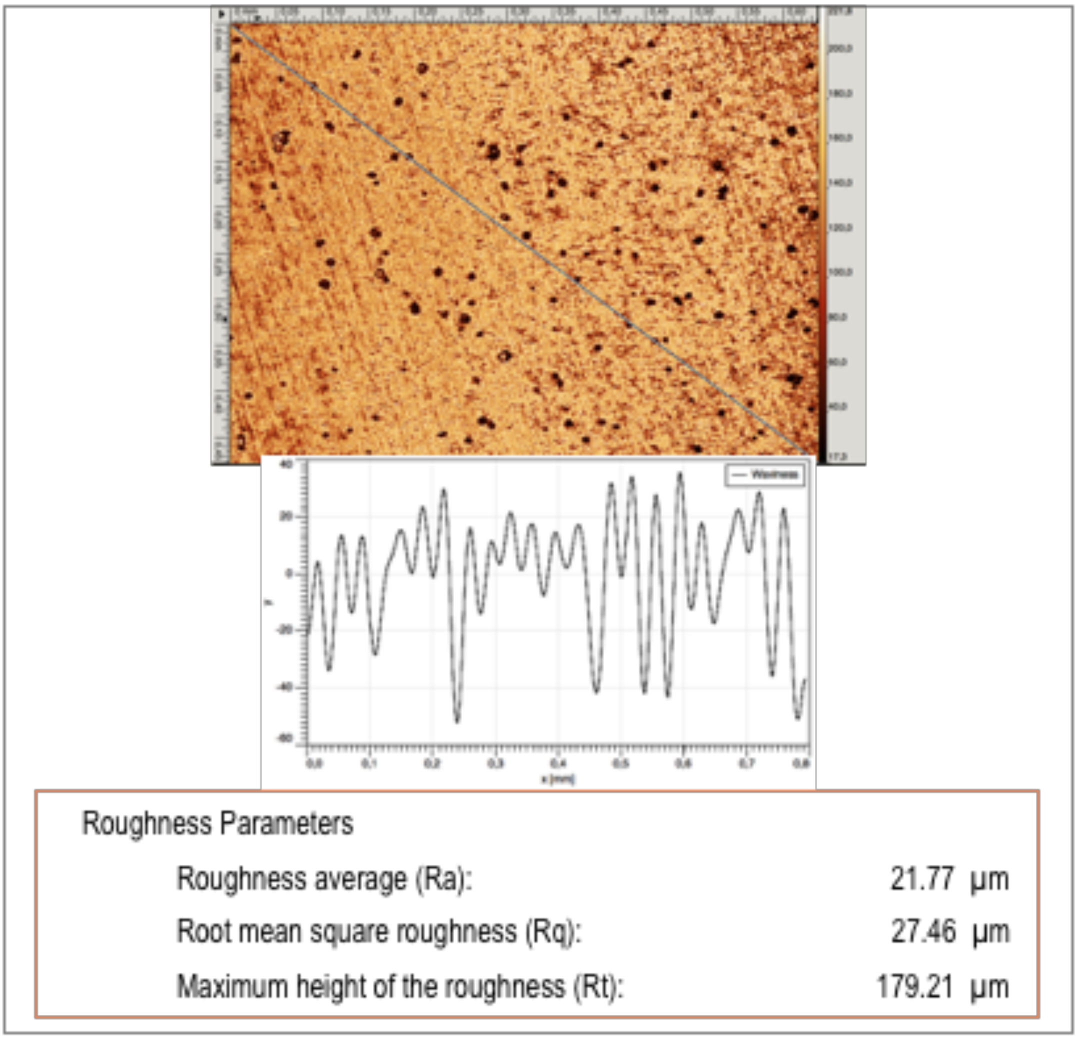


Figure 10. Process of how to obtain roughness parameters in Gwyddion.

To measure the surface roughness of the anodized samples it has been chosen to calculate the parameter Ra (Arithmetic mean roughness). This parameter is defined as 'the arithmetic mean of the absolute values of the coordinates of the points of the roughness profile relative to the Midline within the measurement length [19].

Then, in order to measure the diameter and length of the nanotubes, the function 'Extract profiles along arbitrary lines' was used. With this function the measurements are taken from a profile that you mark in the image. In Figure 11, it is observed how from the profile created of one of the nanotubes, the diameter and length measurements are extracted.

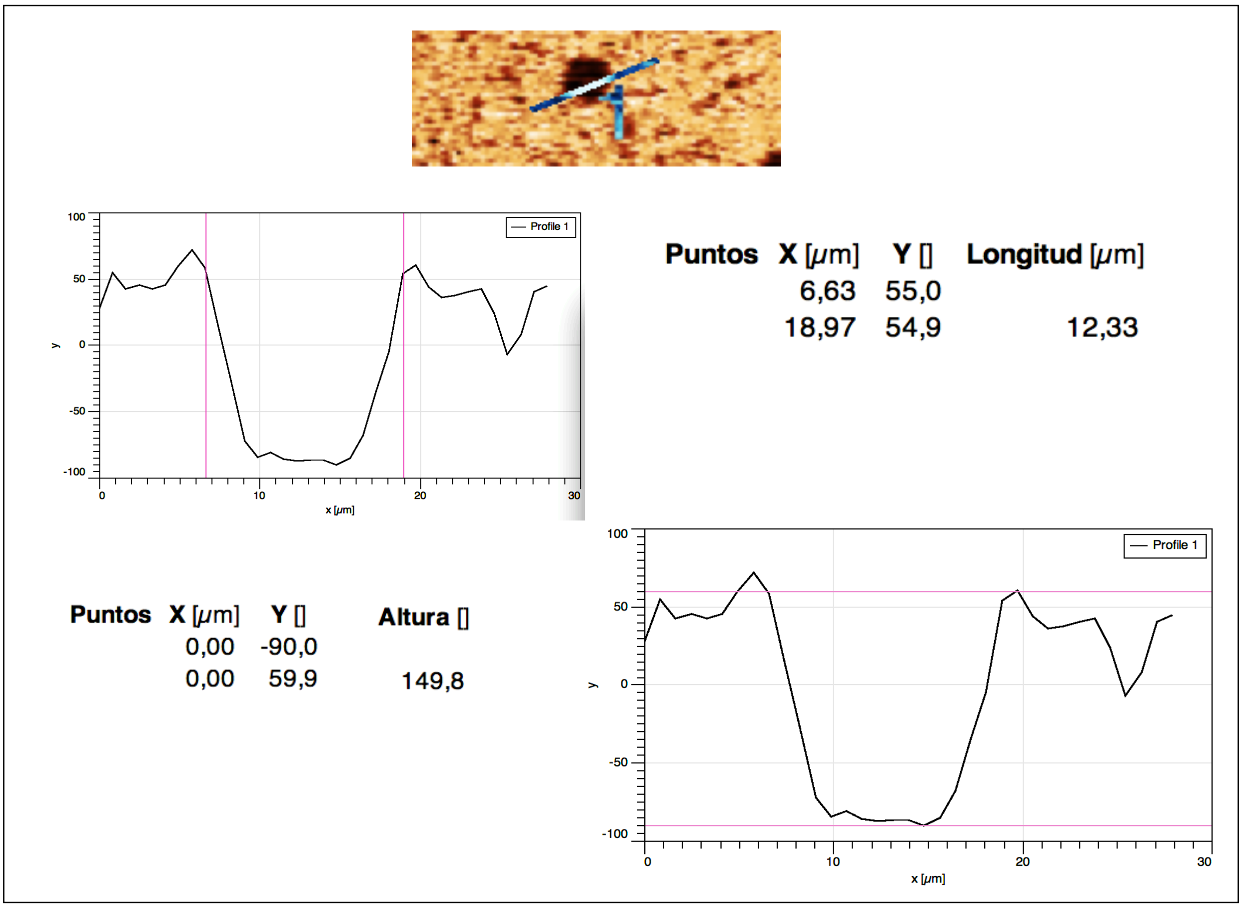


Figure 11. Process of how to obtain diameter and length of nanotubes in Gwyddion.

Finally, the 3D view of the sample can be obtained. This is shown in Figure 12.

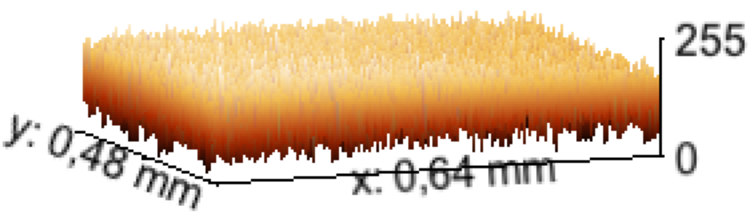


Figure 12. 3D View of the sample obtained by Gwyddion.

5.3. Cytotoxicity assay

The surface of the implants will be in contact with the body, so cell viability assays are important to evaluate both qualitatively and quantitatively the effect on cells of the modification made. It is important to analyze the behavior of the cells when are in contact with the sample. For that reason, two different tests have been carried out: MTS and Live/Dead. Both of them are detailed in sections 5.3.2 and 5.3.3, respectively.

5.3.1. Cell culture

In order to carry out cellular viability tests it has been cultivated human osteoblastic cells (HOB). The cell line used was obtained from the primary human osteoblasts (HOB) which were isolated from femoral trabecular bone from a knee joint after an arthroplasty [20].

First it was carried out the culture of the cells. Because the cells were provided frozen, the vial contained dimethylsulfoxide (DMSO) which reduces osmotic stress and is toxic to the cells at more than 4ºC. The protocol consisted of thawing the vial for 1-2 minutes at 37ºC and inside the sterilized hood add to the vial Dulbecco's Modified Eagle Medium (DMEM) + 10% fetal bovine serum (FBS) to dilute the DMSO to a concentration less than 0.4% (v/v). Finally, it was added in the plate together with the same medium prepared previously, for 24 hours.

For 4 weeks, the cells were cultured obtaining a large number of confluent cells on the surface of the plate. Figure 13 shows the confluence of the cultured osteoblasts.

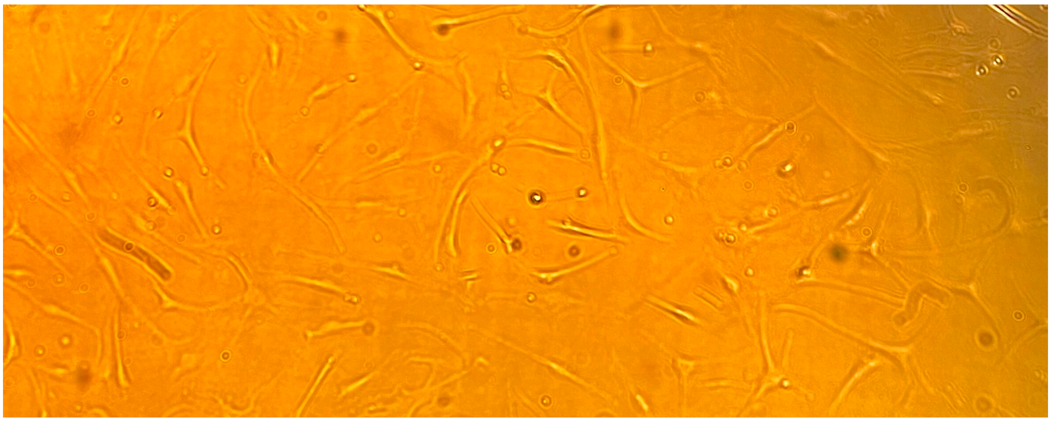


Figure 13. Cultured osteoblasts observed with an optical microscope.

5.3.2.MTS

The MTS assay is a quantitative assay that evaluates the effect of the surfaces studied on cell proliferation and cytotoxicity. In this test the CellTiter 96R Aqueous One Solution Cell Proliferation assay kit (Promega) it has been used and it has been done following the protocol provided by the manufacturer. The CellTiter 96R Aqueous One Solution reagent has been added in sample along with medium. Then, it has been incubated between 1-4 hours at 37ºC with a controlled atmosphere of 5% CO_2. Finally, the absorbance has been read at 490 nm with the Multi-well plate reader Infinite M200 [20].

5.3.3.Phalloidin-tetramethylrhodamine B isothiocynate

Phalloidin-tetramethylrhodamine B isothiocynate assay (DAPI) held to look at the structure of the cells' cytoskeleton and nucleus on the Ti6Al7Nb surface. Phalloidin-tetramethylrhodamine B isothiocyanate kit (Sigma-Aldrich) has been used. To do so, the samples have been cleaned with PBS, and the cells were fixed in a 3.7% solution of formaldehyde (Probus) in PBS for 10 minutes. Then they have been washed with PBS and permeabilized with TRITON X-100 0.1% (Sigma-Aldrich) in PBS for 5 minutes. Finally, the cells were stained with 50 mg/mL of fluorescent phalloidin and 0.2 mg/ml of 4,6-diamidine phenylindole (DAPI) (Sigma-Aldrich) in PBS, for 40 minutes at room temperature protected from light [21].

5.4. Results

5.4.1. Study of the effect of anodization potential and time

The experimental methodology used for the optimization of the anodization conditions, comprised the study of the anodization potential effect (15V, 30V, 45V and 60V) and the immersion time (15', 30', 45', 60') on the rugosity and morphology of the surface.

To reduce the overall cost of the project, the chosen surface characterization method, for a screening among the different conditions, was the confocal interferometry microscopy, due to the free access comparing with high cost of a FESEM (40€/hour).

All the samples were characterized by confocal microscopy and the arithmetic mean roughness (Ra) was determined. The results for the variation of Ra at different potential and anodized times is shown in Graph.

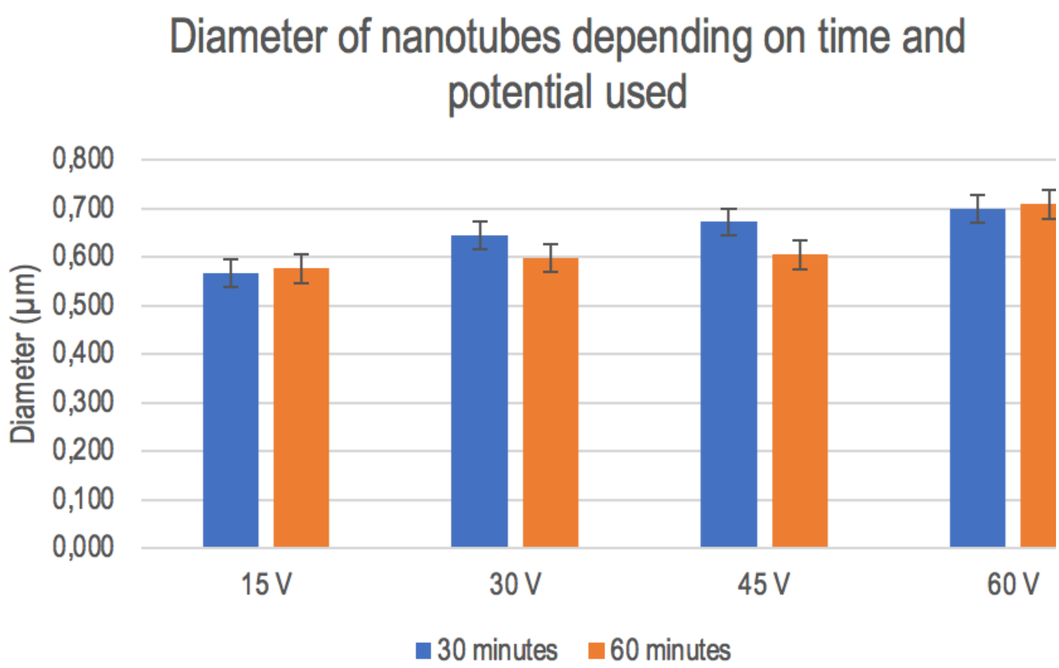
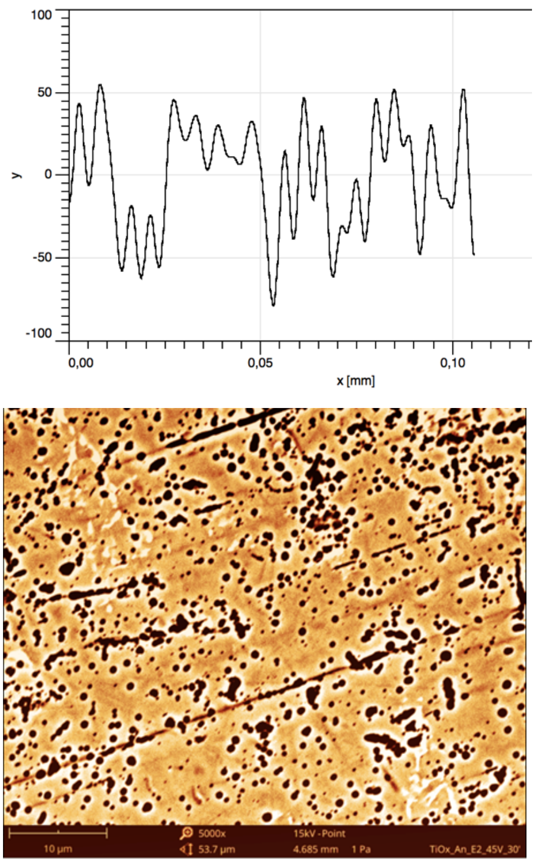


Figure 14. Variation of Ra at different potential and anodized times

As shown in Figure 14, as the potential increases, the roughness of the sample increases as well. In addition, there is no clear effect of an increasing anodization time on roughness.

An example of the imaging of the surface obtained by FESEM microscopy for E2_45V_60' and E2_60V_60' is shown in Figure 15 and Figure 16, respectively.



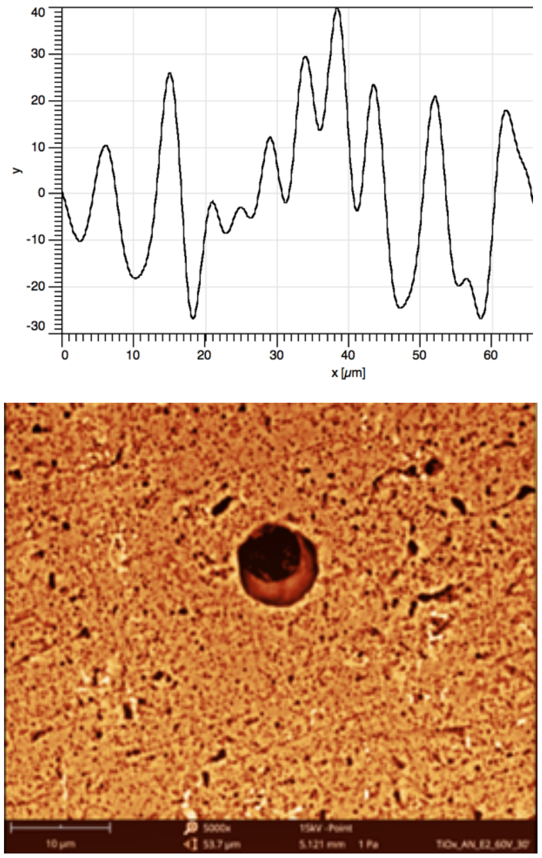


Figure 15. TiOx_AN_E2_45V_60’ **Figure 16.** TiOx_AN_E2_60V_60’

The 3D image of the sample’s surfaces showed small pores on the surfaces. In order to measure the diameter of the observed pores the methodology described in section 5.2.3 was used. Table 2 is an example of the pore diameter measurements by confocal imaging.

	TiOx_AN_E2_ 15V_30’		TiOx_AN_E2_ 30V_30'		TiOx_AN_E2_ 45V_30'		TiOx_AN_E2_ 60V_30'	
NTs	**Ø (µm)**	**STD**	**Ø (µm)**	**STD**	**Ø (µm)**	**STD**	**Ø (µm)**	**STD**
NT 1	0,640		0,674		0,661		0,879	
NT 2	0,650		0,545		0,532		0,557	
NT 3	0,447		0,667		0,873		0,915	
NT 4	0,655		0,634		0,769		0,541	
NT 5	0,582		0,674		0,665		0,759	
NT 6	0,432		0,674		0,532		0,545	
MEAN	**0,57**	0,09	**0,65**	0,05	**0,7**	0,1	**0,7**	0,2

Table 2. Pore diameter for 30 minutes anodization at 15V, 30V, 45V and 60V.

The results of table 2 shows a small increasing in the pore diameter as the potential increases, this result cannot be used to decide the optimal conditions because the low resolution of confocal imaging comparing to the pore diameter size obtained.

The roughness results were used to select the best conditions for the anodization process, 60V during 60 minutes are the optimal conditions for the anodization of Ti-6Al-7Nb samples. The FESEM image of the nanostructured surface is shown in Figure 17.

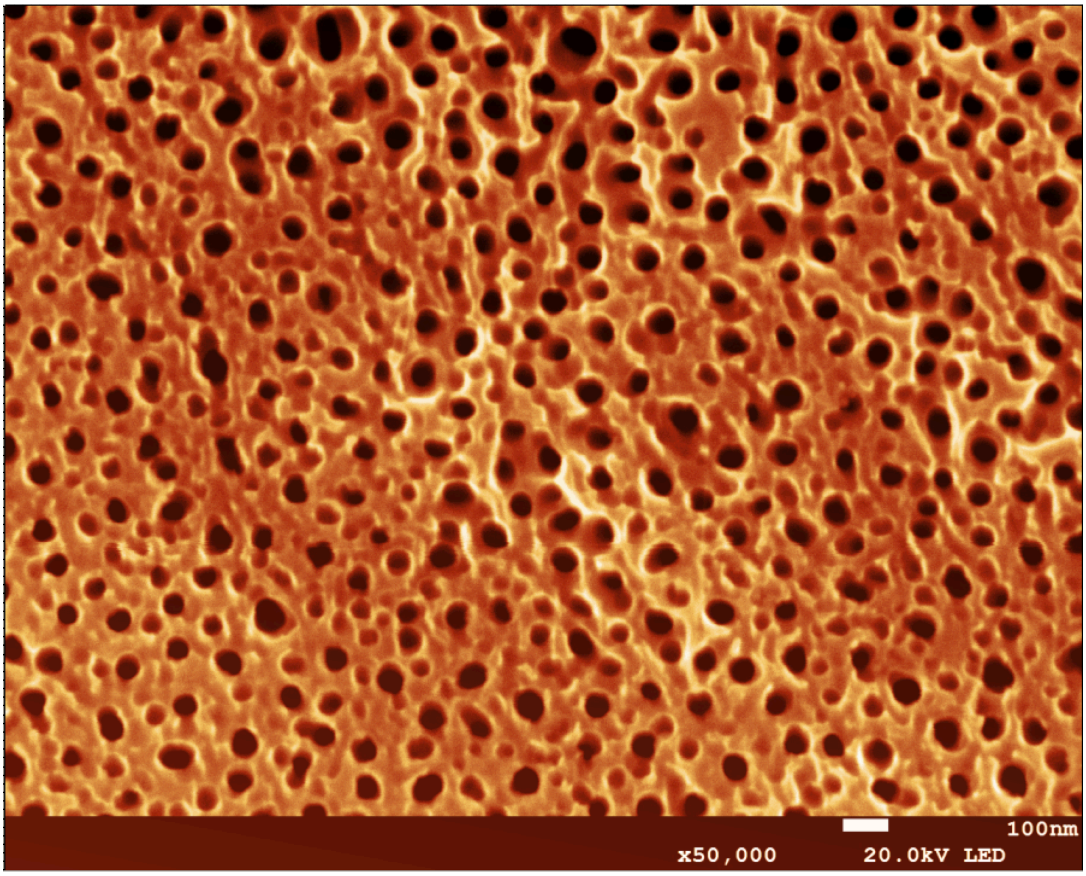


Figure 17. Anodization result obtained with the condition 60 V for 60 minutes

The surface of the samples anodized at 60V for 60 minutes shown a homogeneous surface nanotube structure with diameters of approximately 50 nm of diameter. The dimensions of this nanotubes seem to be suitable to improve the cellular interaction with the implant because the dimensions of this nanostructure are in the same range than the microtubule of the cells.

After the selection of the optimum condition 60V-60', the rugosity achieved was compared with the polished initial substrate to determine the effect of the anodization on the roughness. Table 3 shows the Ra parameter before and after the anodization.

	Ra [µm]	STD
Sample after polishing	1.2	0.2
Sample after anodization	3.9	0.3

Table 3. Ra parameters of samples after polishing and anodizing.

The results observed in Table 4 show a roughness increasing from 1.2 to 3.9 µm. In addition, Figure 18 shows the comparison graph between the roughness profiles of the two samples. Roughness increases considerably after anodizing due to the titanium oxide layer generated.

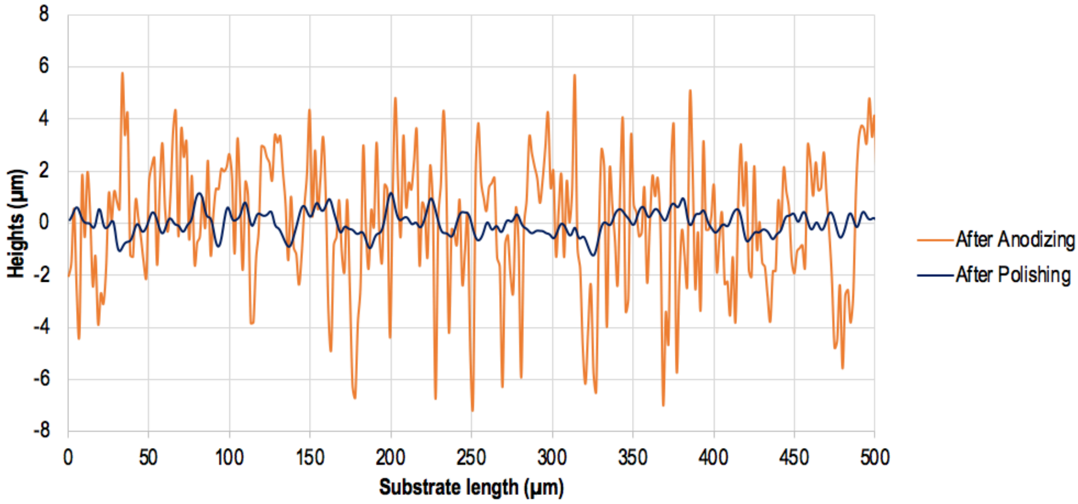


Figure 18. Comparison of roughness after Polishing and after Anodizing.

Figure 19 shows the comparison of the 3D views between a sample after polishing and after anodizing.

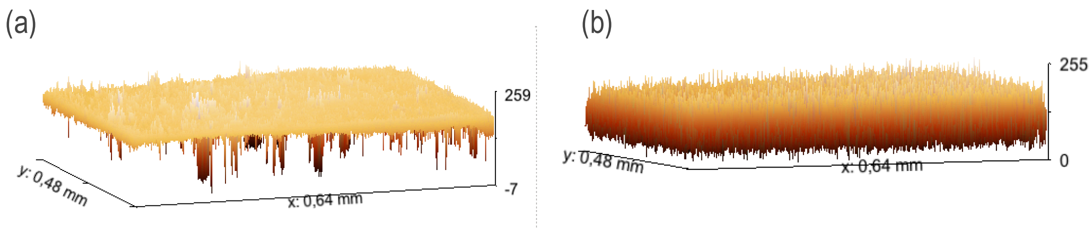


Figure 19. Comparison between 3D views of a (a) sample after polishing and (b) after anodizing.

5.4.2. Diameter of nanotubes

To determine the average diameter of the nanotubes for the sample anodized at 60V-60' the FESEM images have been used. Several images were taken and the diameter for each nanotube was calculated as is shown in Figure 20. Table 4 shows the values obtained for each nanotube in Figure 20.

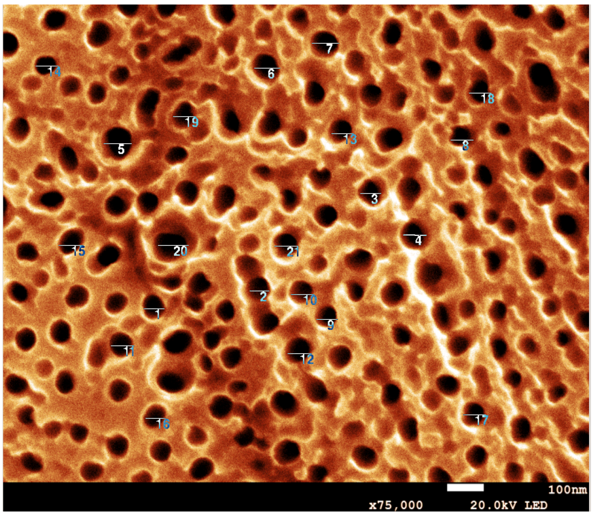


Figure 20. How the diameter values of the nanotubes have been obtained.

	x [nm]		x [nm]
1	54,9899	11	63,5821
2	51,5531	12	58,4268
3	54,9899	13	60,1453
4	73,8927	14	65,3006
5	68,7374	15	53,2715
6	72,1743	16	60,1453
7	60,1453	17	58,4268
8	56,7084	18	60,1453
9	61,8637	19	80,7665
10	61,8637	20	61,8637

Table 4. Diameter of nanotubes observed in figure 20.

For the sample TiOx_AN_E2_60V_30' the average diameter of the nanotube is 59 nm (± 7).

In order to measure the thickness of the oxide layer formed an attempt to do a metallographic preparation of the transversal part of the coating was done. In the observation of the transversal part of the coating by FESEM was not possible to measure the thickness of the coating but it is believed that the duration of anodizing may influence the thickness of the oxide layer.

5.4.3. Anodization of a dental implant

Once the electrochemical anodizing process has been optimized, the protocol has been performed on a commercial dental implant piece (Figure 21). The idea was to understand the effect of the chosen anodization conditions (60V-60') in an 3D complex structure as the dental implants.

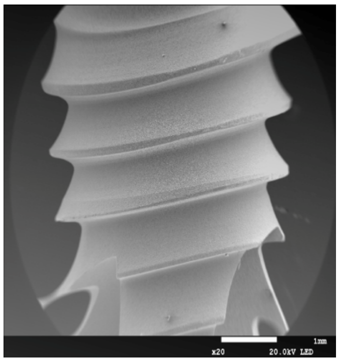


Figure 21. Commercial Dental implant from ®Phibo Dental Solutions

The figure 22 shows a FESEM micrography of the surface of an anodized dental implant at 60V and 60'. The surface of the sample shows a homogeneous distribution of nanotubes on the implant surface (red square). It can also be seen that some nanotube has grown until reach the wall of the next nanotube collapsing and forming a bigger pore (blue square).

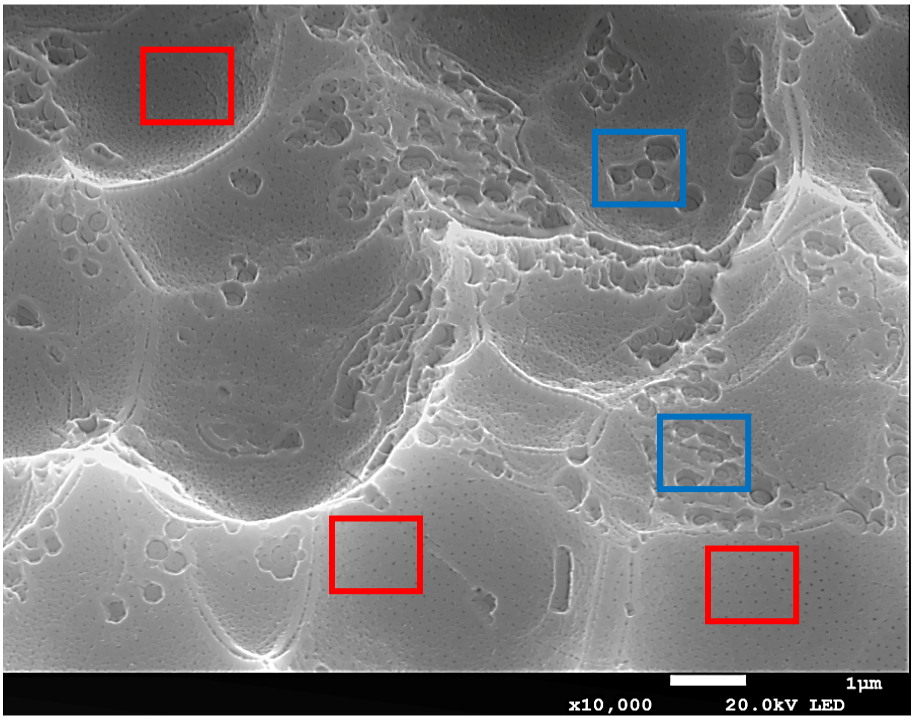


Figure 22. Difference between a medical implant before and after anodization.

5.4.4. Study of electrolyte reuse after an anodization process.

In order to reproduce this experimental procedure in an industrial process it is necessary to know the number of samples that can be anodized with the same electrolyte before is necessary it's renewal. To answer this question, an area of 0,5 cm^2 of Ti-6Al-7Nb sample was anodized in 20 mL of electrolyte solution.

After each sample anodization the electrolyte was analyzed by IR spectroscopy to observe changes in their composition due to the ethylene glycol degradation that may affect the anodization process. Also, the morphology of the samples obtained was analyzed by FESEM to determine the modifications in the nanotube structure.

The Figure 23 shows the pathway of the degradation of ethylene glycol by oxidation process described by Wieland, et al. [22]. The oxidation products of the ethylene glycol degradation are glycolaldehyde, glyoxal, glycolic acid, and so on.

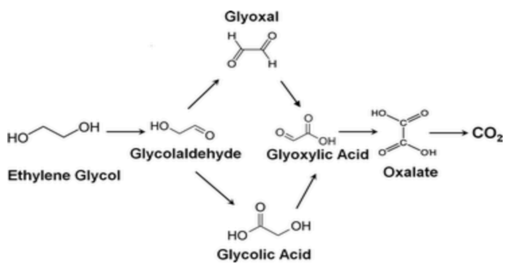


Figure 23. Reaction Pathway of ethylene glycol oxidation onto a platinum electrode [23].

The Figure (24) show the IR spectrum of the electrolyte without use (New electrolyte) and the electrolyte after three anodization of samples.

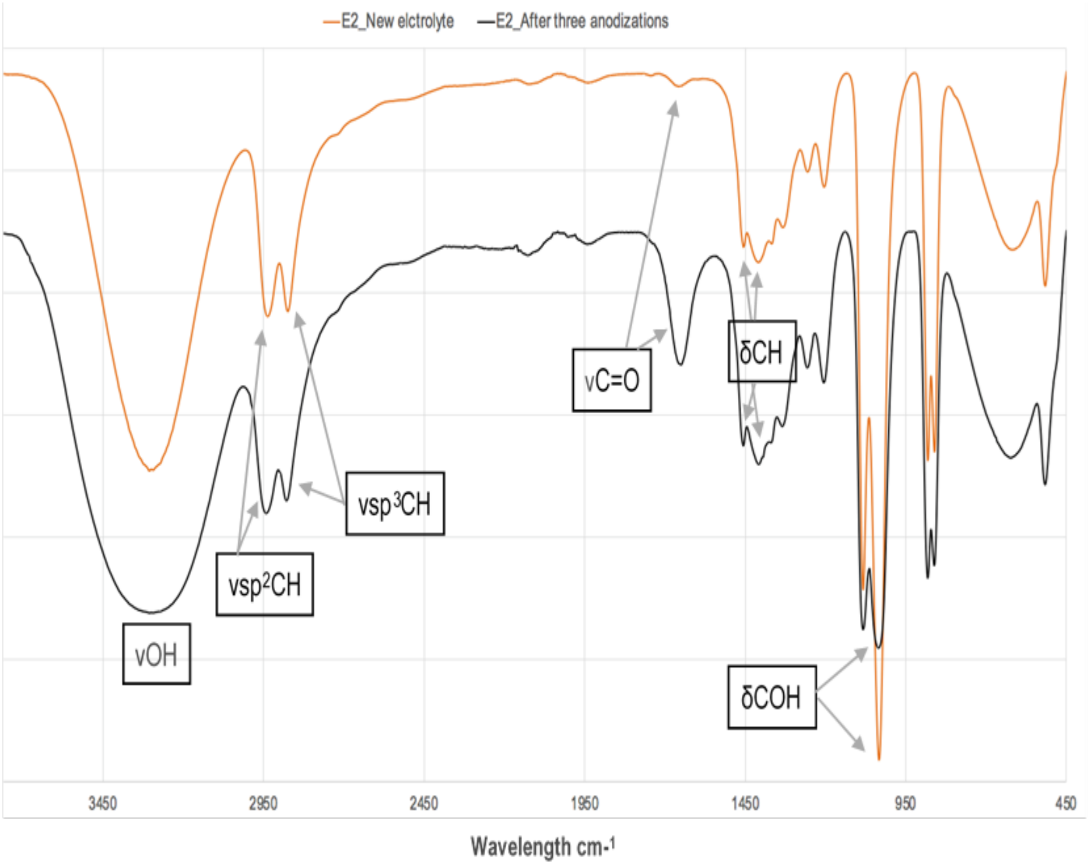


Figure 24. Comparison of IR spectras of new electrolyte and electrolyte after three anodizations.

Almost all the IR bands are coincident between the two spectra, as the stretching O-H band (vOH) from the water molecules in the electrolyte, the C-H stretching for sp^2 and sp^3 carbon (vsp^2CH) and (vsp^3CH), respectively. Also, some other common bands are the bending C-H (δCH) and the bending of the bond C-O-H (δCOH).

The IR spectra of the new electrolyte does not show the characteristic stretching C=O band (vC=O) that only appears in the electrolyte used after the third anodization of a sample. This signal (vC=O) is characteristic from the degradation products of the ethylene glycol as glycolaldehyde, glyoxal and so on.

Comparing the IR results with the obtained on FESEM images (shown in Figure 25), where it can be seen the surface morphology after the use of a new electrolyte after three different anodization suggests that is not possible to reuse the same electrolyte to anodize more than 2 samples to avoid the degradation of the electrolyte damage the nanotube morphology.

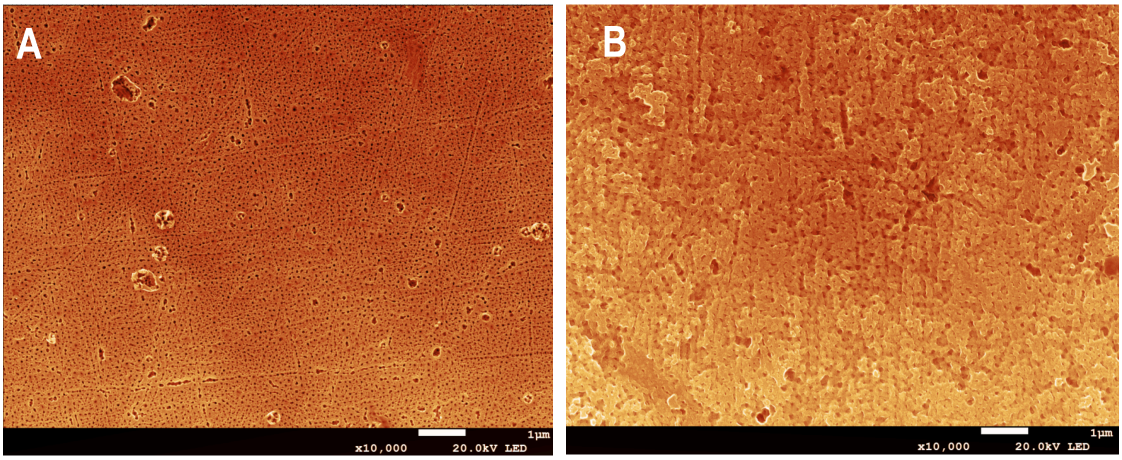


Figure 25. Comparison of FESEM images between a sample anodized using a new electrolyte (A) and an electrolyte used after three different anodizations (B).

Once the maximum number of anodizing processes that could be performed with the electrolyte was determined, around 30 samples were performed with the condition of 60 V for 60 minutes to continue with the cytocompatibility studies.

With this condition and changing the electrolyte every two or three samples, the desired result was obtained: a surface with homogeneously distributed nanotubes with a similar diameter.

5.4.5. Cytotoxicity assay

As regard the results of cytotoxicity tests, the following results have been obtained. First of all, quantitative results from MTS assay have shown (Figure 26). Then the images obtained from DAPI assay are shown in Figure 27.

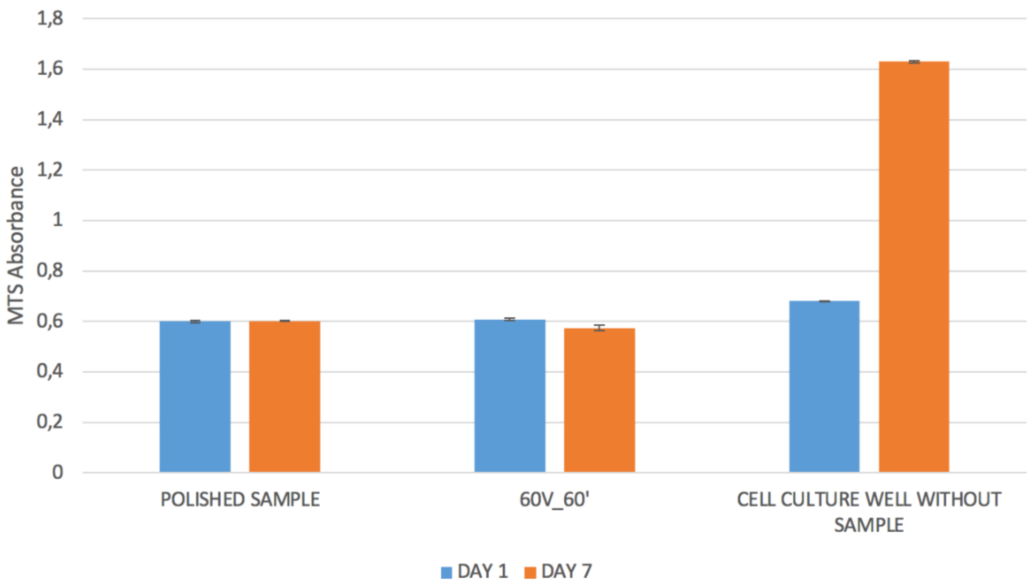


Figure 26. MTS assay results.

As can be seen in Figure 26, the samples do not present cytotoxicity. The absorbance obtained at 1 day in the samples of both polished and anodized titanium and the control (cell culture well without sample) do not present significant differences. At 7 days there is an increase in proliferation in the sample without metal substrate due to the availability of more area available for adhesion, this fact has increased proliferation in samples without metal substrate.

The increased signal at 7 days in cell culture well without titanium sample is due to the fact that metal substrates limit the area for cell adhesion, so are not allowed to proliferate. For that reason, an improvement to the assay is to do it with metal substrates with a larger available area.

On the other hand, the results obtained in the DAPI assay, has allowed to qualitative observation of the cells' cytoskeleton and nucleus on the Ti6Al7Nb surface.

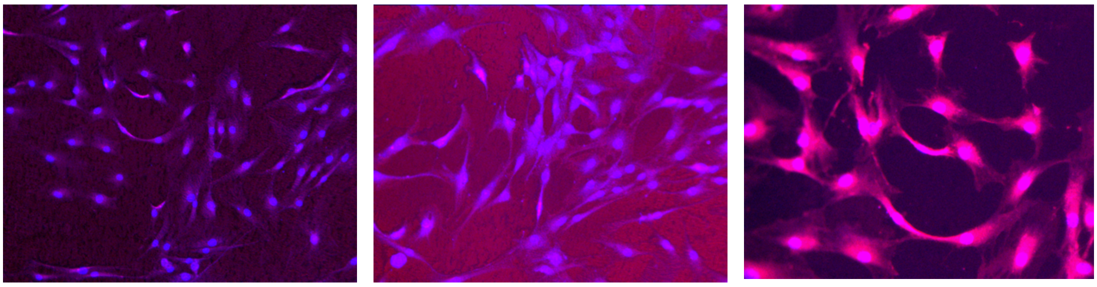


Figure 27. DAPI assay results after 7 days culture.

After 7 days culture the human osteoblastic cells are spreading onto the anodized titanium surface presenting an elongate shape. The Dapi/Phalloidin staining results shows no cytotoxicity.

6. TIMETABLE OF IMPLEMENTATION

In this section the schedule of the execution of this project will be organized. The tasks carried out in this project are distributed and an analysis of the time spent in each of them is made. To do this, it has been made an EDT or WBS (Work Breakdown Structure), a PERT (Project Evaluation and Review Techniques) and a Gantt diagram.

6.1. EDT or WBS

The following image (Figure 28) shows the basic deliverables of this project and its corresponding activities. The EDT scheme has been divided into five main processes: the previous study of information, the experimental part, the analysis of the results, the completion and review of the project and finally, the presentation.

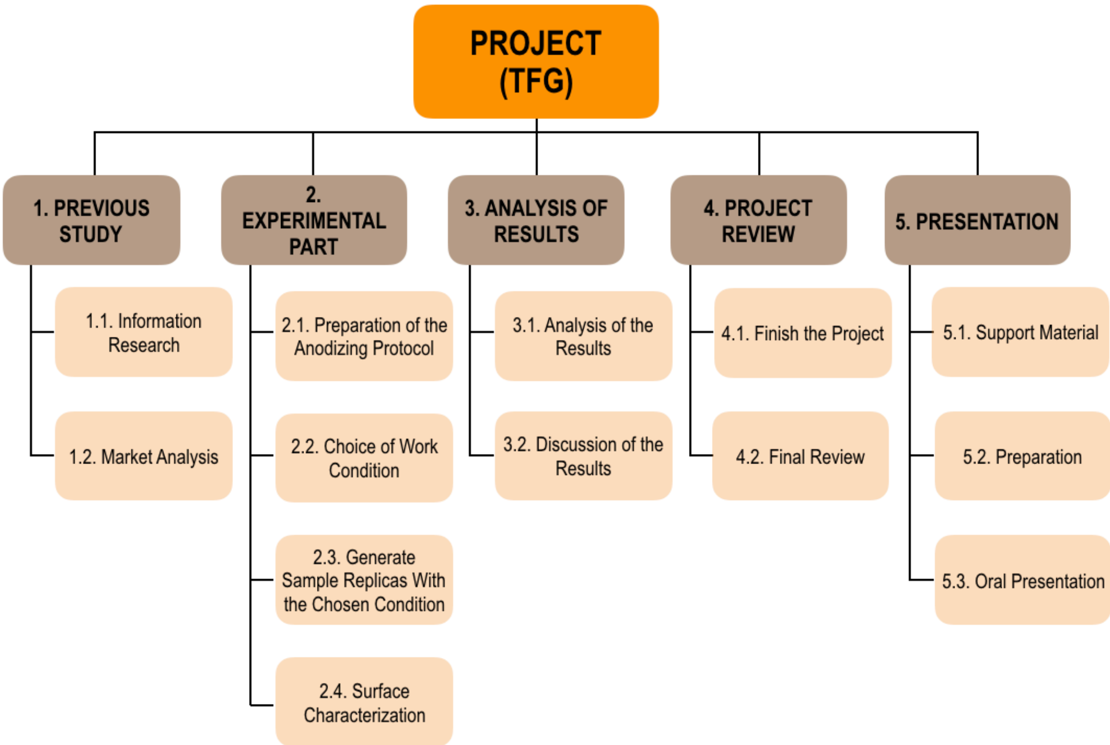


Figure 28. EDT chart where the activities required to carry out the project are specified.

In addition, a dictionary will be performed specifying which tasks each activity involves and the hours that are required to perform them.

ID 1.1	**Control account** 1	**Latest update** 10-06-2021
Description: Information research. Titanium structure, titanium alloys, implant history, implant surface treatments.		
Acceptance criteria: The information must come from reliable and proven sources.		
Deliverables: Introduction, Backgrounds and part of the Experimental section of the final project.		
Assumptions: -		
Assigned resources: Two weeks of the author's work.		
Duration: 40 h		

ID 1.2	**Control account** 1	**Latest update** 10-06-2021
Description: Market analysis of titanium and implants.		
Acceptance criteria: The analysis should show the true character of the market and determine the gap and opportunities.		
Deliverables: Market analysis of the final project.		
Assumptions: -		
Assigned resources: Two days of the author's work.		
Duration: 8 h		

ID 2.1	**Control account** 2	**Latest update** 21-12-2020
Description: Preparation of the anodizing protocol. Develop the sample preparation, cleaning and anodizing protocol.		
Acceptance criteria: Obtain samples with the anodized surface homogeneously.		
Deliverables: A written development has been provided in which the whole procedure to be followed in the experiment is explained.		
Assumptions: Have performed information search.		
Assigned resources: Three months of the author's work and the cost of the material needed.		
Duration: 240 h		

ID 2.2	Control account 2	Latest update 4-02-2021
Description: Choice of work condition. Perform anodized samples with all defined conditions and their replicates. Make images and process the results to choose with which condition to continue.		
Acceptance criteria: Obtain 3 replicas of each condition and in the images characterize the nanotubes generated.		
Deliverables: Processed images and presentation to show the results and choose the condition with the project tutor.		
Assumptions: Have done the design of the protocols.		
Assigned resources: Two months of the author's work and the cost of the material needed.		
Duration: 160 h		

ID 2.3	Control account 2	Latest update 04-04-2021
Description: Generate sample replicas with the chosen condition.		
Acceptance criteria: Obtain 30 samples anodized at 60 V for 60 minutes by changing the electrolyte every three samples.		
Deliverables: 30 samples anodized.		
Assumptions: The condition must be chosen.		
Assigned resources: Two weeks of the author's work and the cost of the material needed.		
Duration: 40 h		

ID 2.4	Control account 2	Latest update 04-06-2021
Description: Surface characterization. Perform images with confocal microscope and FESEM, process images, estimate sample roughness, nanotube diameter and cell viability tests.		
Acceptance criteria: Obtain all the parameters needed.		
Deliverables: Results and graphs of the tests made.		
Assumptions: The samples needed must be done.		
Assigned resources: Two months of the author's work and the cost of the material needed.		
Duration: 160 h		

ID 3.1	Control account 3	Latest update 12-06-2021
Description: Analysis of the results of mechanical and cell viability tests.		
Acceptance criteria: All results should be analyzed.		
Deliverables: The results written and analyzed.		
Assumptions: The experimental part must have finished and the results collected.		
Assigned resources: Three weeks of the author's work.		
Duration: 60 h		

ID 3.2	Control account 3	Latest update 12-06-2021
Description: Discussion of the results.		
Acceptance criteria: All results should be discussed.		
Deliverables: The discussion written.		
Assumptions: The results must be analyzed.		
Assigned resources: One day of the author's work.		
Duration: 5 h		

ID 4.1	Control account 4	Latest update 12-06-2021
Description: Finish the project.		
Acceptance criteria: Finish writing the work.		
Deliverables: The final project.		
Assumptions: All results must be discussed.		
Assigned resources: One day of the author's work.		
Duration: 8 h		

ID 4.2	Control account 4	Latest update 12-06-2021
Description: Final review.		
Acceptance criteria: Complete reading of the project to detect errors and correct them.		
Deliverables: Final project.		
Assumptions: The project must be finish.		
Assigned resources: A week of the author's work.		
Duration: 20 h		

ID 5.1	Control account 5	Latest update 18-06-2021
Description: Support material.		
Acceptance criteria: Develop a dynamic and explanatory presentation of the project.		
Deliverables: Presentation.		
Assumptions: The project must be finished.		
Assigned resources: One day of the author's work.		
Duration: 5 h		

ID 5.2	Control account 5	Latest update 20-06-2021
Description: Preparation of the presentation.		
Acceptance criteria: Prepare a script for the presentation and practice it many times.		
Deliverables: -		
Assumptions: The support material of the presentation must be finished.		
Assigned resources: A week of the author's work.		
Duration: 20 h		

ID 5.3	**Control account** 5	**Latest update** 21-06-2021
Description: Oral presentation.		
Acceptance criteria: Make a good oral presentation in front of the court.		
Deliverables: -		
Assumptions: The preparation of the presentation must be done.		
Assigned resources: One day of the author's work.		
Duration: 1 h		

6.2. PERT

Table 6 summarizes the table which allows to determine the progress of the project and thus be able to develop more easily the corresponding PERT diagram. It can be observed the line of work that has been followed, as well as the time that each task needs. Moreover, it shows the order in which the tasks have been performed. Besides, the critical path can be determined.

Identifier	Activity	Previous activity	Consequential activity	Pessimistic time	Average time	Optimistic weather	PERT time
1.1	A	-	C	42	40	38	40
1.2	B	-	C	10	7	6	7,67
2.1	C	A, B	D	250	240	230	240
2.2	D	C	E, F	160	150	140	150
2.3	E	D	G	42	40	38	40
2.4	F	D	G	160	150	140	150
3.1	G	E, F	H	65	60	60	61,67
3.2	H	G	I	7	5	4	5,33
4.1	I	H	J	9	8	6	7,67
4.2	J	I	K	24	20	18	20,67
5.1	K	J	L	6	5	4	5
5.2	L	K	M	25	20	15	20
5.3	M	L	-	1	1	1	1

Table 4. Activity matrix showing dependency and optimistic, pessimistic and average time.

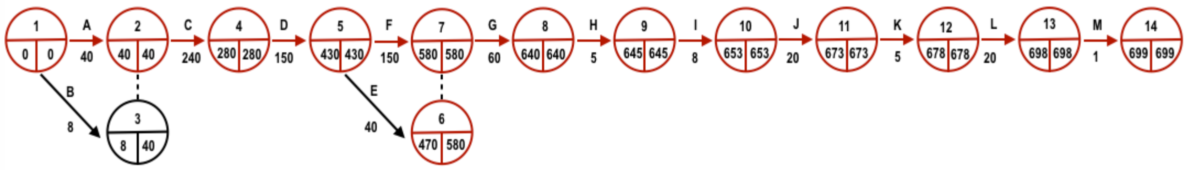


Figure 29. PERT chart where it can be observed the critical path marked in red. The project lasts a total of 699 hours.

6.3. GANTT Chart

The GANTT chart allows to set the course of the project in more detail. We can observe the time periods established for each activity. It is intended to use to organize the development of the project and to be able to finish it within the established timeframe. It will be divided into the tasks of Activity 1, which is the information research that was started at June of 2020. And the experimental part made since October of 2020 until June of 2021. It can be seen in Figure 30.

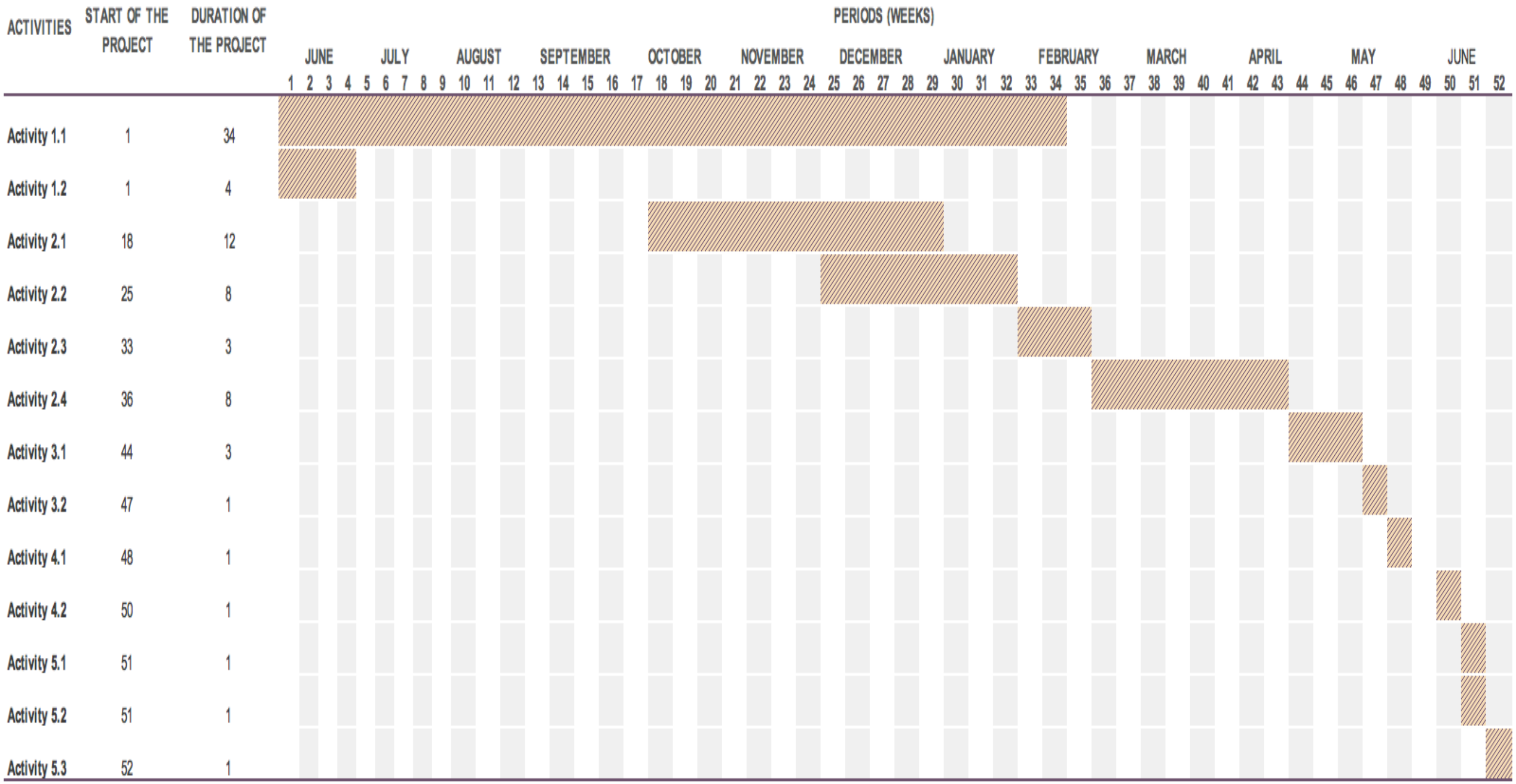


Figure 30. GANTT chart to set the course of the project in more detail.

7. TECHNICAL FEASIBILITY STUDY

In this project, it is necessary to analyze all the internal and external aspects and decide if is technically feasible. For this purpose, SWOT studies are carried out. Moreover, it has been studied the industrial feasibility in order to ensure if the implementation of the electrolytic process industrially is possible.

7.1. Industrial feasibility

One of the objectives of the project is the optimization of the conditions to create nanotubes on the surface of implants in an industrial level. This has been done through a process of electrolytic anodizing controlling potential and time. Electrolytic processes are commonly used at an industrial level, and are made in large electrolytic baths.

Currently, the most important application of titanium anodized are spinal implants. There are many companies that already work with this process: Aesculap Implant Systems, Alphatec Spine, Camber Spine Technologies or Medtronic are examples of how it is possible to implement in an industrial level [24]. The process used is very similar to the one followed in this project, although the electrolytes used are different [25]. For that reason, it can be determined that the implementation of the electrolyte anodizing process for medical implants is feasible in the industry because it is currently already done.

7.2.Strength, Weaknesses, Opportunities and Threats (SWOT)

In this section it is necessary to take into account all aspects that may affect our project. It is a research project with qualified and specialized personnel working on it, using specific machinery, expensive materials and biological samples. In addition, it must be kept in mind that it is a market that needs a lot of research and that it will be necessary in the coming years due to the current global situation. Due to the increase in the use of orthopedic prostheses, the field of medical implants and all its lines of research to improve their bioactivity has great prospects for the future.

Therefore, it has been studied the strengths, weaknesses, opportunities and risks associated with the project through the SWOT.

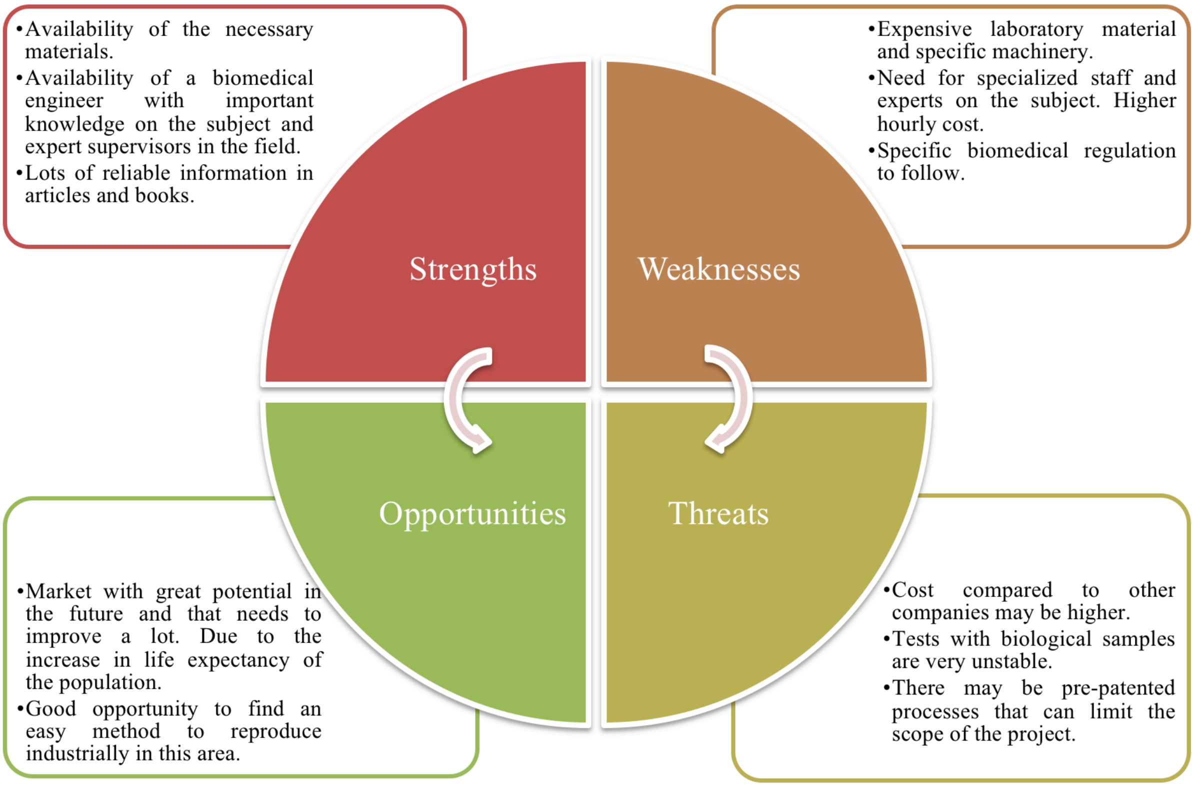


Figure 31. SWOT (Strength, Weaknesses, Opportunities and Threats) of our project.

8. ECONOMIC FEASIBILITY STUDY

In this section an analysis of the economic feasibility of the project is made in order to be able to estimate whether it is feasible to carry it out. When considering the overall project budget, several factors and resources have been taken into account. Thus, these expenses have been divided on the creating nanotubes spend, the characterization spend and the human resources. It is important to explain that when it is tutored by a department of a public entity such as the University of Barcelona, the costs fall on it.

In the economic study it is necessary to consider the material used to carry out the project. Since obtaining the titanium alloy Ti-6Al-7Nb, clean the samples an create de nanotubes by anodizing. Followed by the characterization of the samples using the confocal microscope and FESEM. Moreover, it must be added the human resources of the project depending on all the hours spent by the professionals.

8.1. Creating nanotubes costs

Create nanotubes by the anodizing technique involves different steps. The first step is to obtain a high amount of the alloy Ti-6Al-7Nb and its subsequent preparation to have the samples with the desired measurements. The three-step cleanup protocol must then be performed. In each of the steps, are necessary reagents obtained by the department. Finally, the anodizing of the samples is carried out. The electrolyte used for this final step should be changed often, this is why has been made a great expense of ethylene glycol.

As summarized in Table 5, the total cost of each of the three steps followed to obtain the bioactive surface is 382,2 €.

Creating nanotubes	Quantity (process)	Price per process (€/process)	Total (€)
Ti-6Al-7Nb alloy	5	25	125
Cleaning protocol	1	134.2	134.2
Anodizing	1	123	123
Total			**382.2**

Table 5. Estimated investment for creating nanotubes.

8.2. Characterization costs

As regards the characterization resources to consider, the operative equipment costs which correspond to imaging technique sessions such as Field Emission Scanning Electron Microscopy (FESEM) or Confocal Microscopy, required for the study analysis and sample imaging, have been summarized as seen in Table 6. FESEM is located on the Scientific and Technologic Centre affiliated to the University of Barcelona (CCiTUB) and the cost per session has been more feasible thanks to the existing agreement between institutions [26].

Characterization	Hours (h)	Price per hour(€/h)	Total (€)
Confocal microscopy	10	10.33	103.3
FESEM	5	73.6	368
Total			**471.3**

Table 6. Estimated investment for characterization of samples.

8.3. Human resources costs

As regards the human resources costs, it is considered the hours worked by a recent graduated biomedical engineer. Because of the lack of experience, and taking into account that the average of a salary is usually between 25,000 and 30,000 euros per year [27] it has been estimated a cost of 20 € per hour. On the other hand, the supervisor has a great experience for which the cost of his work has been estimated at 40 € per hour.
As summarized in Table 7, the total cost of human resources is estimated on 15.600 €.

Human resources	Hours (h)	Price per hour(€/h)	Total (€)
Biomedical Engineer	700	20	14000
Supervisor	40	40	1600
Total			**15,600**

Table 7. Estimated investment for human resources.

In order to get the total approximate amount invested in this project, the different costs mentioned previously have been summarized in Table 8:

TOTAL INVESTMENTS	Total (€)
Creating nanotubes	382.2
Characterization	471.3
Human resources	15,600
Total	**16,453.5**

Table 8. Estimated total investments.

So the total investment is approximately 16,453.5 €. It has to take into account that in the actual study the human costs of the biomedical engineer has been obviated because is for educational purposes. In addition, the supervisor's work is not paid at this same price, because the hours employed does not provide any income to her.
Finally, it is important to mention the clear profitability of the project. It is still in research phases but in long term and with an extensive research to corroborate it, it can be implemented in the biomedical sector and can be of great interest.

9. REGULATIONS AND LEGAL MATTERS

This section details the legal aspects and standards to be followed during the implementation of this project. It is important to take into account the place where it is made to conform to the regulations of that country or community and the type of material it is. In this case medical implants are modified. Therefore it is necessary to distinguish what type of medical device it is and explain the regulations to be followed.

9.1. Applicable legislation: rules and regulations

Medical devices are classified by the FDA into three types and are regulated based on their risk and the evaluation needed to demonstrate safety and efficacy. In the case of medical implants, they are class III products because they are long-term implantable devices. These devices require clinical studies that evaluate the safety and efficacy of the device, called pre-market approval application (PMA) [28].

More specifically, the regulation of medical devices in Spain consists mainly of three Royal Decrees corresponding to Community directives and which have been issued in development of the General Law on Health and the Law on Medicinal Products, currently replaced by the Law on Guarantees and Rational Use of Medicines and Health Products [29]. The two Royal Decrees related to are:

- Royal Decree 1616/2009 of 26th October regulating active implantable medical devices.
- Royal Decree 1591/2009 of 16th October regulating medical devices

If the work is a success, it could be possible to market with it, as a Class III medical product [28]. To carry out it will be necessary to take into account these points:

- EC Declaration of Conformity: the CE marking is an express statement that the product complies with all the essential requirements and conformity assessment procedures applicable to it. And it should appear expressly on the product or labeled.
- Certificates of notified bodies: Notified Bodies issue certificates corresponding to the procedures they have followed for the evaluation of products.
- Operating license of manufacturers and importers: manufacturers must have a pre-operating license document issued by the Spanish Agency for Medicines and Healthcare Products.

- Registration of market-based managers: the company has to file a communication with the Spanish Agency for Medicines and Healthcare Products with the company's data and the relationship of products for which it is responsible.

- Declaration of distribution and/or sales activities: companies must file a declaration in the Autonomous Community where they are residing.

In addition, due to its direct contact with the body and the problems in which it may lead, it would be recommended to comply with the main ISO quality and reliability standards in medical products:

- ISO 10993: Biological evaluation of medical devices [30].
- ISO 13485: Quality management for medical devices [31].
- ISO 14971: Application of risk management to medical devices [32].

Depending on the implant performed, it can be supplemented with the ISO standards corresponding to each type of implant.

10. CONCLUSIONS AND FUTURE LINES

About the conclusions of the project, it is important to analyze whether the defined objectives have been achieved or not in order to have a global perspective of the study.

- Have been observe that as the anodization potential increases, the diameter of the nanotubes increases too.
- The optimal conditions to obtain a homogeneous titanium dioxide coating of nanotubes is 60 V for 60 minutes.
- The bioactivity assay shown that the anodized substrate does not present cytotoxicity against human osteoblastic cells.

As a suggestion to continue improving this project and possible future lines:

- A broader study of bioactivity is needed using samples with a larger area to corroborate the increase on bioactivity.
- Mechanical and chemical properties of the substrate should be tested.
- Would be interesting to study is the ion substitution: change the composition of the electrolyte used adding ions to improve the cellular interaction with the substrate. In this case, ions could be added into the structure of the nanotubes.

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